RAW264.7 cells lack prostaglandin-dependent autoregulation of tumor necrosis factor-α secretion

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Abstract **Studies of the response of RAW264.7 cells (RAW) to lipopolysaccharide (LPS) were carried out to determine why these cells do not demonstrate the prostaglandin (PG)-dependent autocrine regulation of tumor necrosis factor-α (TNF-α) secretion observed in primary resident peritoneal macrophages (RPMs). The major cyclooxygenase (COX) product of LPS-stimulated RAW was PGD_2, with lesser amounts of PGE_2. LPS-treated RAW produced PGs more slowly and reached their maximal PG synthetic rate later than did LPS-treated RPMs, as a result of lower constitutive COX-1 expression and a slower rate of COX-2 induction. Cytosolic phospholipase A_2 and levels of free arachidonic acid were similar in RAW and RPMs. In contrast to RPMs, LPS-treated RAW produced high quantities of TNF-α, which were not altered in the presence of COX inhibitors. This failure of endogenous PGs to suppress TNF-α secretion was explained by the absence of the prostaglandin D_2 receptor and the low levels of PGE_2 produced during the first 2 h of the LPS response. These studies demonstrate that autocrine regulation of TNF-α secretion in response to LPS is greatly facilitated by a COX-1-mediated rapid accumulation of PGs as well by a correspondence between the PGs produced and the receptors expressed by the cells.**—Rouzer, C. A., A. T. Jacobs, C. S. Nirodi, P. J. Kingsley, J. D. Morrow, and L. J. Marnett. **RAW264.7 cells lack prostaglandin-dependent autoregulation of tumor necrosis factor-α secretion.** ***J. Lipid Res.*** **2005.** 46: **1027–1037.**

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Cyclooxygenase (COX; prostaglandin G/H synthase) catalyzes the first two steps in the biosynthesis of prostaglandins (PGs), prostacylin, and thromboxane from arachidonic acid (20:4). Initially, the COX activity of the enzyme mediates the *bis*-dioxygenation of 20:4 to the hydroperoxyendoperoxide, PGG_2. Then, the peroxidase activity of COX reduces the hydroperoxy group of PGG_2 to a hydroxyl group, yielding PGH_2. Terminal synthase enzymes, acting on PGH_2, produce all of the other PGs (1–4). Two isoforms of COX have been characterized and have been found to share 60% sequence identity, with nearly identical three-dimensional structures and highly similar active sites (5–9). The kinetics of the consumption of 20:4 by the two isoforms are virtually indistinguishable (10). However, because of minor differences in the active sites, the COX-2 isoform can use neutral ester and amide derivatives of 20:4 that are used only poorly by COX-1 (11–13). The active site differences also have allowed the development of selective inhibitors for each isoform (14–18).

Despite the similarities in structure and kinetics, the two COX isoforms differ considerably with regard to transcriptional regulation. COX-1 is usually constitutively expressed and is found in many different tissues. In contrast, the expression of COX-2 is primarily limited to the immune and nervous systems, where it is induced in response to a variety of inflammatory and proliferative stimuli, such as cytokines, bacterial products, growth factors, and tumor promoters. As a consequence of these varied expression patterns, COX-1 is believed to be primarily responsible for "housekeeping" functions, such as gastric cytoprotection, regulation of platelet aggregation, and modulation of renal function. In contrast, COX-2 is thought to be involved in the inflammatory response and in the control of cellular proliferation (19–23).

Macrophages play a crucial role in the inflammatory and immune responses and are major sources of inflam-

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Abbreviations: ATCC, American Type Culture Collection; COX, cyclooxygenase; $cPGI_2$, carbaprostacyclin; $cPLA_2$, cytosolic phospholipase A_2; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LPS, lipopolysaccharide; mPGES-1, microsomal prostaglandin E synthase-1; PG, prostaglandin; PGI_2, prostacyclin; RAW, RAW264.7 cells; RPM, resident peritoneal macrophage; TNF-α, tumor necrosis factor-α; 20:4, arachidonic acid.

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matory mediators, including PGs. These cells have long been used as models to study the PG synthetic response to a wide variety of stimuli. Because COX-2 is believed to be the primary isoform responsible for inflammatory PGs, much attention has been focused on the induction of COX-2 expression in macrophages in response to inflammatory cytokines and bacterial products. These stimuli typically evoke a concomitant PG synthetic response that begins after 2–4 h and continues for up to 24 h, a pattern often referred to as "delayed" PG synthesis. Until recently, it has been widely accepted that delayed PG synthesis is totally COX-2-dependent, but we have recently shown that murine resident peritoneal macrophages (RPMs) express high levels of COX-1 constitutively and that this isoform contributes significantly to the delayed PG synthesis invoked in these cells in response to bacterial lipopolysaccharide (LPS). Furthermore, PGs generated early in the LPS response, predominantly by COX-1, cause an autocrine suppression of tumor necrosis factor-α (TNF-α) secretion (24). These data call into question the clear distinction between the functional roles of COX-1 and COX-2 in at least some macrophage populations.

Studies of macrophage physiology have been notably advanced by the availability of cell lines such as RAW264.7 (RAW) (25). These cells, derived from pristane-elicited murine peritoneal macrophages transformed with Abelson leukemia virus, have been particularly valuable because of their ease of culture, rapid growth rate, and phenotypic resemblance to primary macrophages. Despite these similarities, we have discovered that RAW differ significantly from RPMs in their response to LPS. In particular, we have found qualitative, quantitative, and kinetic differences in the PG synthetic response of LPS-treated RAW versus RPMs. Also notable is our finding that RAW produce much greater quantities of TNF-α in response to LPS than do RPMs, primarily because they fail to demonstrate PG-dependent autocrine regulation of TNF-α secretion. Because RAW have practically become synonymous with macrophages in the study of cellular physiology, it is important to understand and recognize the ways in which these cells differ from primary macrophage populations. Consequently, we report here a comprehensive study of the LPS response in RAW to develop an understanding of the mechanisms underlying the differences noted between these cells and primary murine RPMs.

EXPERIMENTAL PROCEDURES

RAW culture

RAW (25) were obtained from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle's medium supplemented with GlutaMax, high glucose, sodium pyruvate, and pyridoxine-HCl (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Summit Biotechnologies, Fort Collins, CO). Cells were plated onto 35 mm tissue culture dishes to provide monolayers at 35–45% confluence for experimental incubations. The mean protein content of RAW cultures at the time of the experiments was 170 ± 20 μg/dish ($1.4 \pm 0.1 \times 10^6$ cells/dish).

LPS treatment of RAW cultures

Cultures of RAW were washed twice with PBS at 37°C and then overlaid with 1 ml of fresh, serum-free DMEM. After a 1 h incubation, an additional 1 ml of medium with or without 200 ng/ml LPS (*Escherichia coli* 011:B4; Calbiochem, San Diego, CA) was added. Cells were incubated for the desired time periods. For the determination of COX-1, COX-2, cytosolic phospholipase A_2 ($cPLA_2$), and microsomal prostaglandin E synthase-1 (mPGES-1) expression as well as PG synthesis, the medium was collected from each culture and the cells were then washed twice with ice-cold PBS. Cell monolayers were scraped into 200 μl of lysis buffer [50 mM Tris-HCl, pH 7.5, plus 150 mM NaCl, 4 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.2% Triton X-100, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/ml each of antipain, leupeptin, chymostatin, and pepstatin (all components from Sigma, St. Louis, MO)]. Cell lysates were allowed to stand for 30 min on ice with occasional vortex mixing, and particulate material was then removed by centrifugation for 10 min at 16,000 *g*. Samples of culture medium and cell lysates were stored at −80°C until analyses could be completed. For the measurement of free 20:4, the medium was removed from the cell cultures and combined with 1 ml of ice-cold acetonitrile containing 100 ng of 20:4-d_8 (Cayman, Ann Arbor, MI). The cells were scraped twice into a total volume of 1 ml of ice-cold methanol, and the resulting cell lysate was added to the medium solution. In some experiments, the medium was collected and placed in one tube containing 1 ml of the internal standard solution, and the cell lysate in 1 ml of methanol was placed into a separate tube containing 1 ml of the internal standard solution. This allowed separate determination of lipid levels in the medium versus cells.

For evaluation of the effects of selective COX inhibitors, cells were incubated in serum-free medium containing 100 nM of the COX-2 inhibitor SC-236 and/or the COX-1 inhibitor SC-560 (both from Calbiochem) for 1 h before LPS addition. Incubation with LPS was then carried out in the ongoing presence of the inhibitors. The inhibitors were added as 100 μM stock solutions in DMSO, and the DMSO concentration in all cultures was maintained at 0.1% for these experiments. For the evaluation of the effects of exogenous PGs, the desired concentrations of PGE_2 and/or carbaprostacyclin ($cPGI_2$; both from Cayman) were added to RAW cultures simultaneously with LPS. PGs were prepared as stock solutions in DMSO, and the DMSO concentration in all cultures was maintained at 0.1%.

Immunoblotting for protein expression

The protein concentrations of 20 μl aliquots of RAW lysates were determined using a BCA Protein Assay kit (Pierce, Rockford, IL) according to the manufacturer's directions. Macrophage lysate samples containing 15 μg of protein were then subjected to SDS-PAGE using an 8% gel (12% for mPGES-1) overlaid with a 3% stacking gel. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA), and the membranes were processed as described (24). After the membranes were overlaid with ECL detection reagent (Amersham, Piscataway, NJ), the chemiluminescence signal intensity was measured using a Fluor-S Max Multi-Imager (Bio-Rad, Hercules, CA). The membranes were also exposed to hyperfilm ECL film (Amersham) to obtain photographic images.

Assay of PGs

PG levels in culture medium were determined by negative ion GC-MS of pentafluorobenzyl ester derivatives as previously described (26) or by selected reaction monitoring of the ammoniated ions by positive ion electrospray ionization liquid chromatography/tandem mass spectrometry (LC/MS/MS) (Kingsley, P. J., and L. J. Marnett, unpublished observations).

Determination of total 20:4 levels

The LC/MS/MS-based assay of 20:4 in RAW medium and/or cells was carried out as described (24, 27).

Assay for TNF-α

The concentrations of TNF-α in cell culture medium were determined by an OptEIA assay kit (PharMingen, San Diego, CA) according to the manufacturer's instructions.

CRE-luciferase transcription assay

RAW were seeded at a relative density of 2×10^5/well in a six-well dish. Cells were cotransfected overnight using the Effectene® reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions with 0.4 μg/well of a luciferase reporter construct driven by the cAMP-responsive element (Clontech, Palo Alto, CA) and 0.4 μg/well of a cytomegalovirus (CMV) promoter-driven Renilla luciferase construct (Promega, Madison, WI). Cells were rinsed with serum-free medium before a 6 h treatment with either vehicle or agonist in serum-free medium. Cell lysates were processed using the Dual Luciferase Assay® kit (Promega) according to the manufacturer's protocol. Values from CRE-driven luciferase expression were normalized to those obtained from CMV-driven Renilla luciferase expression.

RT-PCR assay for PG receptor expression

Total RNA was isolated from RAW, NIH 3T3 cells, and mouse lung tissue using the RNeasy total RNA isolation kit (Qiagen). Briefly, cultured cells, ~80% confluent in 10 cm dishes, were washed once with ice-cold PBS, scraped into ice-cold PBS, and then centrifuged for 5 min at 5,000 *g*. RNA was immediately extracted from the cell pellets according to the RNeasy protocol. For lung tissue, freshly dissected mouse lung from CD-1 mice (Charles River Laboratories, Wilmington, MA) was cut into small sections (~2 mm), immersed in RNAlater (Ambion, Austin, TX), and stored at 4°C. Approximately 30 mg of lung tissue was subsequently removed from RNAlater solution and homogenized in RNA lysis buffer RLT (Qiagen) using a Teflon pestle. RNA was immediately extracted from the tissue homogenate according to the RNeasy protocol. Total RNA was quantified by absorbance at 260 nm (A_{260}), and then aliquots (10 μg) were treated with 10 units of RQ1 RNase-free DNase (Promega) for 30 min at 37°C. RNA was extracted directly from the reaction mix using phenol-chloroform-isoamyl alcohol (25:24:1), pH 6.6 (Ambion), and precipitated in 70% ethanol. RNA pellets were dissolved in nuclease-free water (Ambion) and quantified by A_{260}. A_{260}/A_{280} ratios were all greater than 1.8. First-strand cDNA synthesis and subsequent PCR were performed using Ready-to-Go RT-PCR beads (Amersham). cDNA synthesis was performed on 1 μg of total RNA according to the manufacturer's protocol, and random hexamers were used as the first-strand primer. For negative control reactions (no RT), the RT was first heat-inactivated at 95°C for 5 min. Gene-specific primers (**Table 1**) were used for subsequent PCR procedures, with a thermocycle program of 30 s at 95°C, 30 s at 55°C, 1 min at 72°C for 30 cycles, and a final 5 min extension at 72°C. Amplification products were resolved by 1.5% agarose gel electrophoresis and photographed using the Gel Doc EQ system (Bio-Rad).

RESULTS

Synthesis of PGs by RAW in response to LPS

RAW were incubated in the presence or absence of 100 ng/ml LPS for periods up to 24 h, and the culture medium was analyzed for PGs by GC-MS. RAW secreted primarily PGD_2 and PGE_2, with PGD_2 predominating (**Fig. 1A**). A small but significant increase in PG levels could be detected in the cells as early as 1 h after the addition of LPS. However, a marked increase in the rate of PG synthesis did not occur in RAW until after 2 h, and the maximal rate was not reached until after 4 h (Fig. 1B). After 6 h of LPS incubation, PGD_2 and PGE_2 had reached levels of 1,600 ± 200 pmol/10^7 cells and 310 ± 30 pmol/10^7 cells, respectively. During the ensuing 18 h, PGE_2 levels increased slightly to 400 ± 110 pmol/10^7 cells, whereas PGD_2 levels decreased to 900 ± 120 pmol/10^7 cells. The latter data indicate that the PG synthetic rate decreased markedly after 6 h. Because PGD_2 is chemically unstable, undergoing spontaneous dehydration to PGA_2, its level decreases if the de novo synthetic rate is slower than the rate of decomposi-

TABLE 1. Primers and product sizes for RT-PCR assay of prostaglandin receptor expression

Receptor	Accession Number	Primer Set	Product Size (Sequence)
DP1[a]	NM_008962	5′-GGGGCTTCTGGGCAATCTTCT-3′ 5′-TCCACCGCCATAGCCAACAG-3′	300 (207–506)
EP1	NM_013641	5′-GCTCGGCTGCCACCTTCC-3′ 5′-TGCGCGGGCCACAGATAC-3′	260 (503–762)
EP2	NM_008964	5′-CCTGCCGCTGCTCAACTACG-3′ 5′-GAGCTCGGAGGTCCCACTTTTC-3′	392 (885–1,276)
EP3	NM_011196	5′-CGGCGGGCAACGAGACA-3′ 5′-TATCAATAGCGGCGACCAACAGA-3′	260 (687–946)
EP4	NM_008965	5′-CTTTCGCCGCCGCAGGAGTTT-3′ 5′-CGGGCGAGGAAGGAGCGAGAGT-3′	390 (976–1,365)
FP	NM_008966	5′-AACGGAGGCATAGCTGTCTTTGTA-3′ 5′-TCTTCCCAGTCTTCGATGTGCTCT-3′	344 (454–797)
IP	NM_008967	5′-TTCCGCTTCAACGCCTTCAA-3′ 5′-AGAGCAGCCGTCACCACCAGT-3′	285 (600–884)
TP	NM_009325	5′-TCGGGCTCATATTCGCACTCC-3′ 5′-TGGCCACACGCAGGTAGATGA-3′	303 (762–1,064)
DP2 (CRTH2)	NM_009962	5′-CTGCACCTGGCGCTATCCGACTTG-3′ 5′-ACCGCCAGAGCCCAGAGCATCAG-3′	269 (461–729)

[a] Receptor identities are as follows: DPI-2 are receptors for prostaglandin D_2. EP1-4 are receptors for prostaglandin E_2. FP, IP, and TP are the receptors for prostaglandin $F_{2\alpha}$, prostaglandin I_2, and thromboxane A_2, respectively. DP2 is the receptor for prostaglandin D_2 also designated $CRTH_2$ (chemoattractant receptor homologous molecule on TH2 cells).

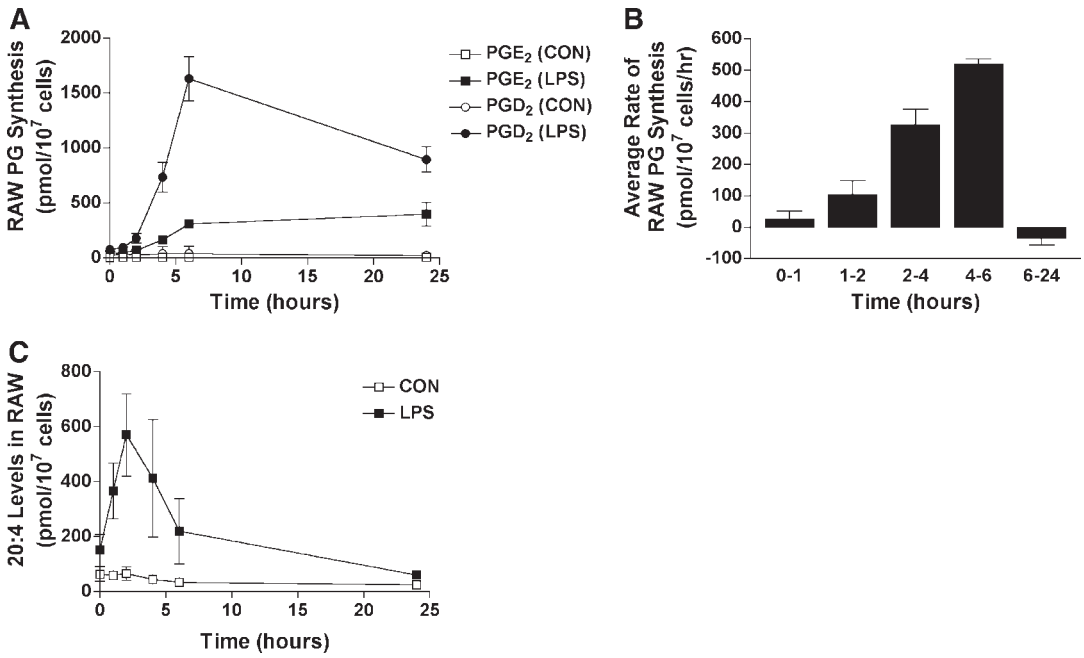

Fig. 1. Time course of prostaglandin (PG) synthesis by lipopolysaccharide (LPS)-stimulated RAW264.7 cells (RAW). A: RAW were plated in 35 mm culture dishes as described in Experimental Procedures and incubated for the indicated times in the presence (LPS) or absence [CON (control)] of 100 ng/ml LPS. The medium was collected from each culture and analyzed for the indicated PGs by GC-MS. B: The data in A were used to calculate the average rate of total net PG synthesis above control (no LPS) levels over the indicated time periods after LPS addition. C: Cultures were prepared and incubated in parallel with those used to obtain the results shown in A. At the indicated times, the medium and cell lysates were combined and analyzed for arachidonic acid (20:4) content by liquid chromatography/tandem mass spectrometry (LC/MS/MS), as described in Experimental Procedures. Each data point (A, C) or bar (B) is the mean ± SD from the combined results of three separate experiments in which duplicate determinations were made.

tion. In contrast, PGE_2 is stable under culture conditions, so that the gradual increase in its levels reflects the slow rate of ongoing PG synthesis. In RAW not treated with LPS, PGD_2 levels remained constant at 20–40 pmol/10^7 cells, and PGE_2 levels remained constant at 7–14 pmol/10^7 cells.

It is notable that RAW differ significantly from RPMs in their PG synthetic response to LPS. The primary PGs produced by RPMs are prostacyclin (PGI_2; the major product) and PGE_2. The PG synthetic rate increases rapidly in RPMs, reaching its maximal value after only 2 h of incubation with LPS. Total levels of PGs produced after 6 h of LPS incubation are 50–260% higher in RPMs than in RAW, ranging from 2,900 ± 800 pmol/10^7 cells to 6,900 ± 1,400 pmol/10^7 cells, depending on the genetic background (24).

Changes in 20:4 levels in RAW during the LPS response

After LPS addition, the total amount of 20:4 in RAW cultures (medium plus cells) increased gradually, peaked at 2 h, and then slowly returned to baseline (Fig. 1C). A similar time course of 20:4 increase was observed in RPMs, although maximal levels of 20:4 reached in RAW (570 ± 150 pmol/10^7 cells) were somewhat higher than those achieved in RPMs (320 ± 90 pmol/10^7 cells) (24). The difference, however, did not reach statistical significance ($P = 0.077$).

The data presented in Fig. 1C reflect levels of 20:4 in the combined medium and cell lysates from RAW cultures. In separate experiments, RAW were incubated for 0, 2, or 6 h in the presence of LPS, and the cells and medium were analyzed separately for free 20:4 content. The results showed that ~44–50% of the free 20:4 was localized to the cells regardless of the period of incubation with LPS. Thus, the maximum intracellular 20:4 content in RAW during LPS incubation reached values of 200–350 pmol/10^7 cells, a value similar to that obtained for RPM cultures (180–300 pmol/10^7 cells), in which a higher fraction (73–77%) of total 20:4 was cell associated (24). Therefore, the somewhat higher 20:4 levels observed in RAW cultures was attributable primarily to free fatty acid in the medium.

Levels of PG synthetic enzymes during the LPS response in RAW

As was observed for RPMs, LPS treatment strongly induced COX-2 expression in RAW (**Fig. 2A, B**), although the time course was somewhat different for the two cell populations. Based on immunoblot signal intensity, LPS-dependent COX-2 expression reached only 8% of the maximum value within the first 2 h in RAW (Fig. 2A, B), whereas in RPMs, 28% of the maximum was reached in this time period (24). Furthermore, COX-2 levels increased throughout the full 24 h incubation in RAW (Fig. 2A, B), whereas in RPMs, they reached a maximum at 6–10 h and decreased thereafter (24). Despite these temporal differences, direct immunoblot comparison of RAW and RPM lysates revealed that maximal COX-2 protein ex-

pression was similar in the two cell types (Fig. 2C, D). Therefore, the increased PG synthetic ability of RPMs compared with RAW cannot be explained on the basis of major differences in COX-2 protein expression, although it should be noted that the immunoblot analysis does not directly measure the activity of the expressed enzyme.

Our previous report (24) indicated that COX-1 plays a significant role in PG formation by LPS-treated RPM. Therefore, we examined the expression of this enzyme in RAW compared with RPMs. Both cell types constitutively express COX-1, and levels were seen to decrease slightly during the 24 h LPS exposure (Fig. 2E, F). However, RPM expression of COX-1 was found to be 8-fold higher (based on immunoblot signal intensity) than that of RAW, a finding that may help to explain the differences in PG synthetic capacity between the two cell types.

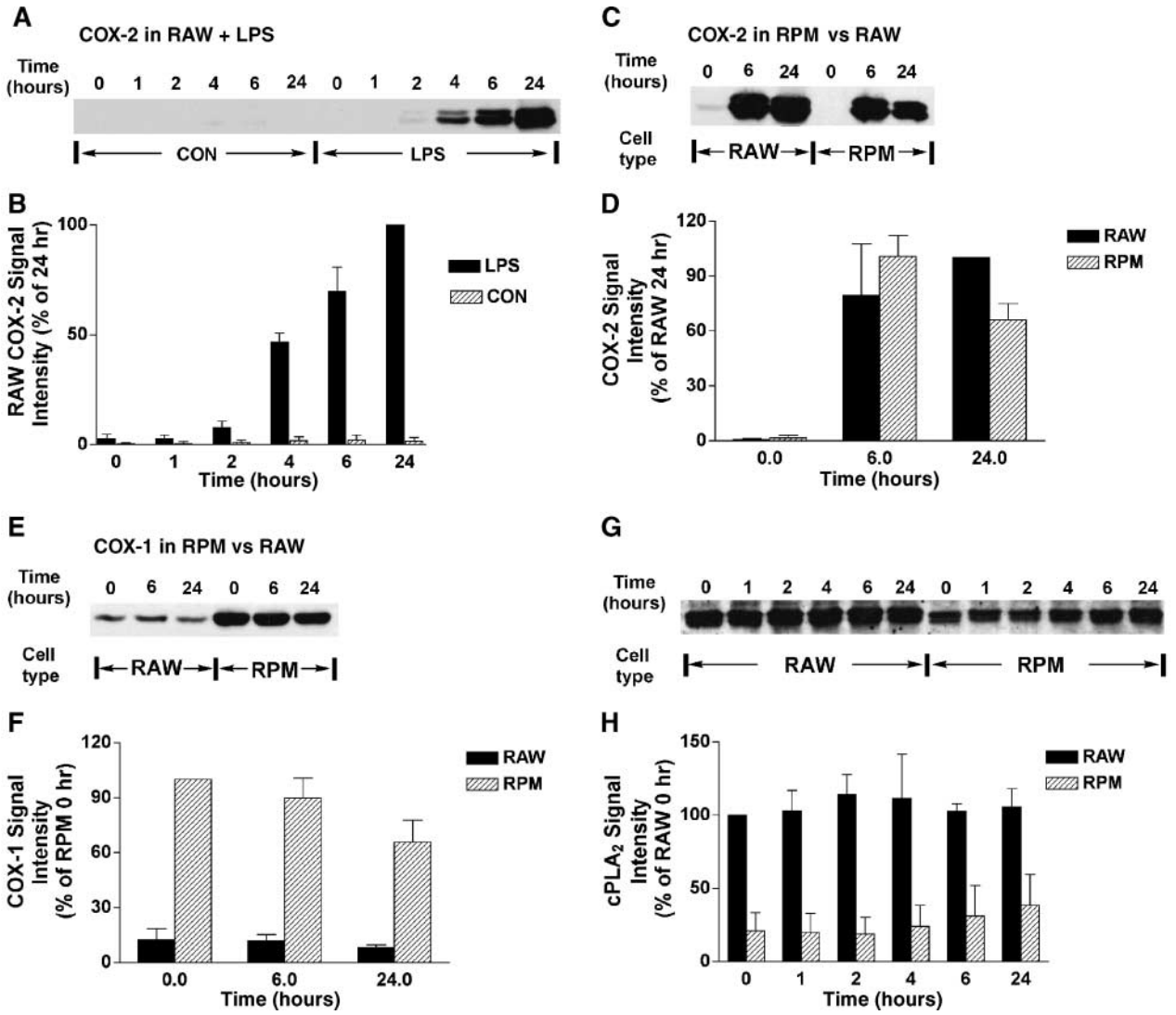

Fig. 2. Expression of 20:4 metabolizing enzymes during the LPS response. A: RAW were plated onto 35 mm dishes as described in Experimental Procedures and incubated for the indicated times in the presence of 100 ng/ml LPS. Lysates (15 μg of protein) prepared from the cell monolayers were subjected to immunoblot analysis to detect cyclooxygenase-2 (COX-2) protein content, as described in Experimental Procedures. The results were visualized by exposure of hyperfilm ECL film. B: A Fluor-S Max Multi-imager was used to quantify the COX-2 chemiluminescence signal from immunoblots prepared as shown in A using cells incubated in both the presence (LPS) and absence [CON (control)] of LPS. The quantitative results for each blot were normalized to the COX-2 signal obtained for cells at 24 h of incubation, and the data were combined to yield means ± SD from three separate experiments in which duplicate samples were analyzed. C, D: Experimental conditions were identical to those described for A, B, except that lysates (15 μg of protein) prepared from resident peritoneal macrophage (RPM) cultures incubated for the indicated times with LPS (24) were included for comparison purposes. The quantitative data in D are normalized to the COX-2 chemiluminescence signal in RAW samples from the 24 h time point, and the results are means ± SD from three separate experiments in which duplicate samples were analyzed. E, F: Experimental conditions were identical to those used for C, D, except that immunoblots were analyzed for COX-1 protein content. The quantitative data in F are normalized to the COX-1 chemiluminescence signal in RPM samples from the 0 h time point, and the results are means ± SD from three separate experiments in which duplicate samples were analyzed. G, H: Experimental conditions were identical to those used for C, D, except that immunoblots were analyzed for cytosolic phospholipase A_2 ($cPLA_2$) protein content. The quantitative data in H are normalized to the $cPLA_2$ chemiluminescence signal in RAW samples from the 0 h time point, and the results are means ± SD from three separate experiments in which duplicate samples were analyzed.

The major enzyme responsible for 20:4 release from cellular phospholipids is $cPLA_2$. In contrast to the results obtained for COX-1, $cPLA_2$ expression was found to be higher in RAW than in RPMs (Fig. 2G, H). Also notable was our finding that $cPLA_2$ levels did not increase in LPS-treated RAW compared with RPMs, in which a small induction was observed. As noted above, 20:4 levels are somewhat higher in LPS-treated RAW than in RPMs, a difference attributable mostly to extracellular 20:4. However, at the time of peak free 20:4 levels (2 h after LPS addition), the expression of $cPLA_2$ in RAW was 6-fold higher than in RPMs (based on immunoblot signal intensity), a difference that was much greater than the modest difference in free 20:4 observed.

In our previous studies, we noted that the relative proportion of PGE_2 versus PGI_2 increased with time in LPS-treated RPMs as a result of the induction of mPGES-1. In contrast, mPGES-1 was not detected in RAW at any time during the LPS treatment (data not shown), a finding consistent with data reported previously (28). Although the relative proportion of PGE_2 versus PGD_2 increases with time in LPS-treated RAW, this is most likely attributable to the chemical instability of PGD_2, as noted above, rather than to increased PGE_2 synthase activity.

Differences between different lots of RAW

Two lots of RAW were available in the Vanderbilt Tissue Culture Core Facility. The cells used for the experiments described above (RAW-1) were acquired from the ATCC in 2001. The second lot (RAW-2) was acquired in the early 1990s. Both lots were stored under liquid nitrogen according to ATCC-recommended procedures. However, despite similarities in handling and identical culture conditions for the present experiments, marked differences were noted in the PG synthetic capacities between these two cell lots. As seen in **Fig. 3A**, RAW-2 responded more slowly and produced much lower total quantities of PGs than did RAW-1 (Fig. 1A), with the major differential being in the quantity of PGD_2. Comparative immunoblot analysis showed much lower constitutive expression of COX-1 (Fig. 3B) and induced expression of COX-2 (Fig. 3C) in RAW-2 compared with RAW-1, although the expression of $cPLA_2$ was similar (Fig. 3D). A third lot of RAW, obtained from the Alliance for Cell Signaling, gave results very similar to those of RAW-1 (data not shown). Because RAW-1 was more recently acquired from the ATCC, and therefore more likely to be representative of cells used in other laboratories, further studies were carried out using this lot.

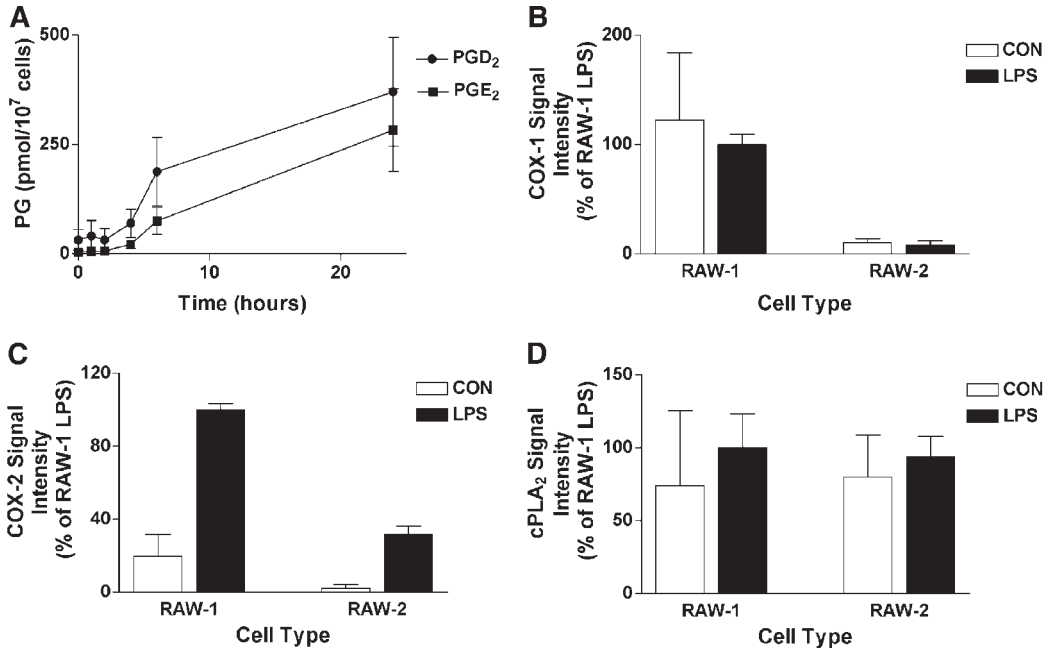

Fig. 3. Comparison of 20:4 metabolism in two separate lots of RAW. A: RAW originally acquired from the American Type Culture Collection (ATCC) in the early 1990s (RAW-2) were incubated with LPS under conditions identical to those described in the legend to Fig. 1A. Levels of PGE_2 and PGD_2 in the culture medium were determined by GC-MS and are means ± SD from the combined results of two experiments in which duplicate determinations were made. B: After a 6 h incubation without [CON (control)] or with LPS (LPS), cell lysates were prepared from the lot of RAW used for the experiments illustrated in Figs. 1, 2 (RAW-1; acquired from the ATCC in 2001) and the lot used for the experiment illustrated in A (RAW-2). Equal amounts of protein from each lot of cells (15 μg) were subjected to immunoblot analysis for COX-1 protein expression. The quantitative results were normalized to the COX-1 chemiluminescence signal in the RAW-1 sample treated with LPS and are means ± SD from the combined results of two experiments in which duplicate determinations were made. C: Conditions were identical to those described in B, except that analysis was carried out for COX-2 protein. D: Conditions were identical to those described in B, except that analysis was carried out for $cPLA_2$ protein.

LPS-induced TNF-α secretion is not inhibited by endogenous PGs in RAW

We recently reported that TNF-α secretion by LPS-treated RPMs is inhibited by endogenously synthesized PGs and that the main source of the inhibitory PGs is COX-1 (24). Therefore, we examined LPS-dependent TNF-α secretion in RAW to determine if this phenomenon would apply to other macrophage populations. LPS-treated RAW secreted TNF-α, with levels beginning to increase at 2 h of incubation and reaching a maximum at 6 h (**Fig. 4A**).

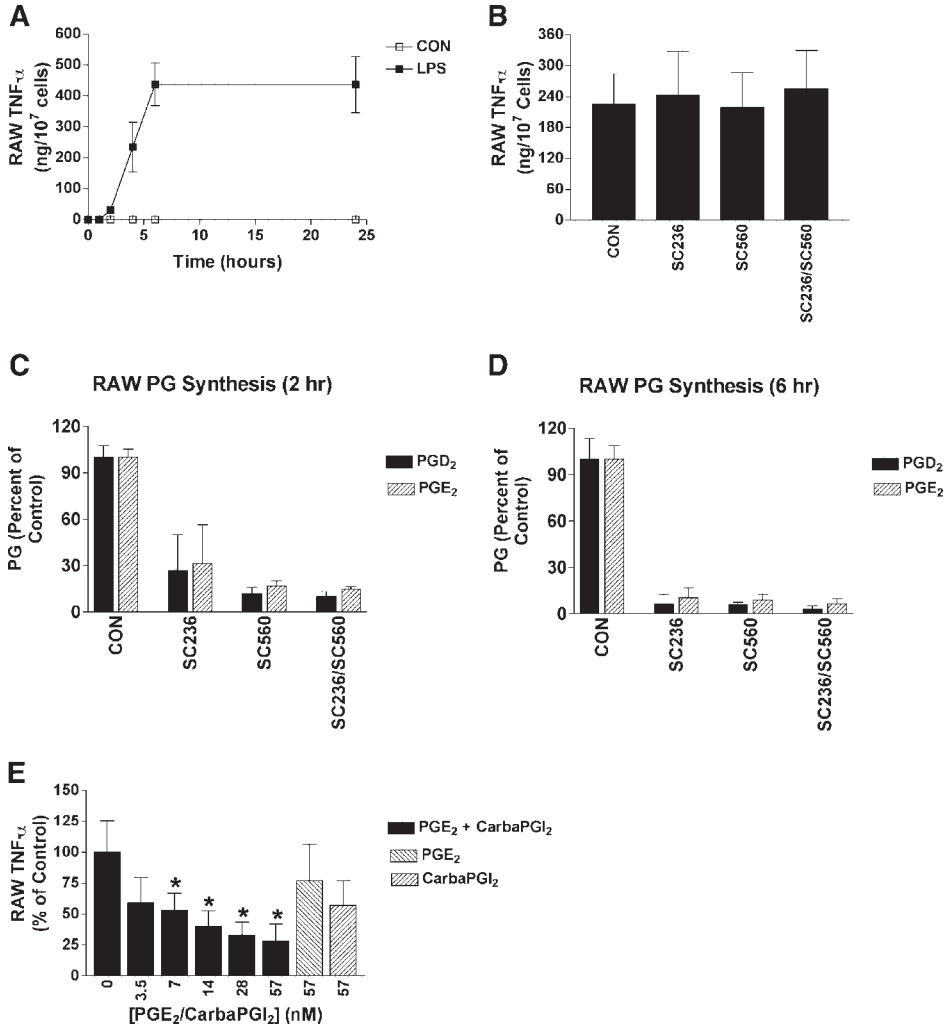

Fig. 4. Tumor necrosis factor-α (TNF-α) secretion in LPS-treated RAW. A: RAW were plated in 35 mm culture dishes as described in Experimental Procedures and incubated for the indicated times in the presence (LPS) or absence [CON (control)] of 100 ng/ml LPS. The medium was collected from each culture and analyzed for TNF-α concentration by ELISA. Values are means ± SD from the combined results of three separate experiments in which duplicate determinations were made. B: RAW were plated in 35 mm culture dishes as described in Experimental Procedures. After a 1 h preincubation in the presence of the indicated inhibitor(s) (100 nM) or vehicle (DMSO), LPS (100 ng/ml) was added and the cells were incubated for 6 h. Levels of TNF-α in the culture medium were determined by ELISA. Results are means ± SD from the combined results of three separate experiments in which duplicate samples were analyzed. C: RAW were plated in 35 mm culture dishes as described in Experimental Procedures. After a 1 h preincubation in the presence of the indicated inhibitor(s) (100 nM) or vehicle (DMSO), LPS (100 ng/ml) was added and the cells were incubated for 2 h. Levels of PGs in the culture medium were determined by LC/MS/MS. Data are expressed as the percentage of control PG synthesis (CON; no inhibitor) achieved in the presence of each inhibitor and are means ± SD from the combined results of three separate experiments in which duplicate samples were analyzed. D: Experimental conditions were identical to those described in C, except that cultures were incubated with LPS for 6 h. E: RAW were incubated for 6 h with LPS (100 ng/ml) in the presence of the indicated concentrations of PGE_2, carbaprostacyclin ($CarbaPGI_2$), or both. PGs or vehicle (DMSO) were added to the cultures at the same time as LPS. Cells were incubated for 6 h, and then the culture medium was harvested for determination of TNF-α by ELISA. Data are expressed as the percentage of the TNF-α concentration in medium from RAW incubated in the absence of any added PG and are means ± SD from the combined results of three separate experiments in which duplicate samples were analyzed. Asterisks indicate that the values were statistically different from those of RAW incubated without added PGs ($P < 0.05$).

Maximal levels of TNF-α in RAW culture medium (440 ± 79 ng/10^7) were much higher than in RPM culture medium (51 ± 20 ng/10^7). Furthermore, although RAW and RPMs reached maximal levels of TNF-α at the same time, those levels were sustained for the full 24 h culture period in RAW cultures, whereas in RPMs they decreased between 6 and 24 h (24).

The markedly higher TNF-α secretion in LPS-treated RAW compared with RPMs suggested the possibility that RAW are not affected by inhibition from endogenously secreted PGs. To test this hypothesis, we investigated the effects of selective COX inhibitors on both PG synthesis and TNF-α secretion. At 100 nM concentrations, neither the selective COX-1 inhibitor, SC560, nor the selective COX-2 inhibitor, SC236, had any effect on TNF-α secretion in LPS-treated RAW (Fig. 4B). This was despite the fact that, after 2 h of LPS exposure, SC236 and SC560 inhibited total PG synthesis by 71% and 86%, respectively, and after 6 h of LPS exposure, both inhibitors effected a 92% suppression of PG synthesis (Fig. 4C, D). Note that the levels of inhibition observed with these compounds at 6 h of incubation are inconsistent with the hypothesis that each affects only the COX isoform to which it is directed. Similar results, which likely reflect a lack of specificity in SC560, were also found with RPMs (24).

Regardless of the specificity of the COX inhibitors, the data clearly show that inhibition of PG synthesis has no effect on TNF-α secretion, a finding very different from that in LPS-treated RPMs. One possible explanation for this difference is that RAW are unable to respond to a PG stimulus with TNF-α synthesis inhibition. However, when RAW were incubated with LPS in the presence of increasing concentrations of PGE_2 and $cPGI_2$, a stable analog of PGI_2, up to a 70% inhibition of TNF-α formation was observed (Fig. 4E). Very similar results were obtained in LPS-treated RPMs, in which endogenous PG synthesis was inhibited by SC560 (24). Thus, it appears that both cell types have similar abilities to respond to PGs with TNF-α synthesis inhibition.

The data in Fig. 4E demonstrate that PGs of the type and in the quantities produced by RPMs inhibit TNF-α secretion in LPS-treated RAW. Yet RAW produce PGD_2 instead of the PGI_2 produced by RPMs. Both cell types produce PGE_2, and RAW responded to PGE_2 in combination with $cPGI_2$, but PGE_2 alone was not highly effective in these cells (Fig. 4E). Therefore, a possible explanation for the failure of RAW to respond to their endogenous PGs with TNF-α synthesis inhibition is that PGD_2 cannot replace PGI_2 for TNF-α synthesis inhibition. PG-mediated inhibition of TNF-α secretion has been shown to be dependent on increases in intracellular cAMP, a response mediated by PGE_2 through the prostaglandin E_2 receptors (EP2 and EP4), and by PGD_2 through the DP1 receptor (29–33). Therefore, we tested the ability of RAW to respond to PGE_2 and PGD_2 with cAMP accumulation by transfecting cells with a luciferase reporter plasmid driven by the cAMP-responsive element. The cells were then incubated in the presence of forskolin, butaprost (a synthetic EP2 receptor agonist), BW245C (a synthetic DP1 agonist), PGE_2, and PGD_2. The results showed strong luciferase activity in response to forskolin and the EP receptor agonists but no activity in response to BW245C or PGD_2 (**Fig. 5**). These data are consistent with prior reports showing that RAW express both the EP2 and EP4 receptors and suggest that they lack a functioning DP1 receptor.

To confirm the results shown in Fig. 5, we performed RT-PCR on mRNA isolated from RAW to characterize the full complement of PG receptors. The results demonstrated the presence of mRNA for EP1, EP2, EP4, the prostaglandin I_2 receptor (IP), and the thromboxane A_2 receptor (TP), findings totally consistent with the response of these cells to PGE_2 and $cPGI_2$. No mRNA for DP1 was detected (**Fig. 6**), although the message was demonstrated in the total RNA from mouse lung (Fig. 6). Although mRNA for the DP2 (CRTH2) receptor was observed in both RAW and NIH 3T3 cells, this receptor is believed to be coupled to G_i and would therefore evoke decreases rather than increases in cAMP (34). Together, these results indicate that RAW cannot respond to endogenous PGD_2 with TNF-α synthesis inhibition because they do not express the requisite DP1 receptor.

DISCUSSION

In this study, we investigated LPS-mediated 20:4 metabolism and TNF-α secretion in the RAW264.7 cell line, allowing a direct comparison with our previous results from primary RPMs. Our findings demonstrate that, although there are basic similarities in the responses of the two cell types, there are also significant temporal and quantitative differences. Specifically, RAW produce lower total quantities of PGs in response to LPS than do RPMs, and the maximal rate of PG synthesis is delayed in RAW compared with RPMs. We also find that the primary PG produced by

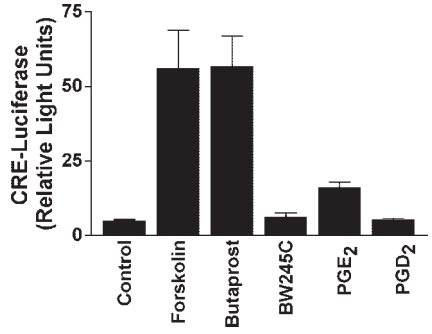

Fig. 5. Failure of prostaglandin D_2 receptor (DP) agonists to activate CRE-driven luciferase gene expression in RAW. RAW (2×10^5) were cotransfected with 0.4 μg each of CMV-driven Renilla and the CRE-driven firefly luciferase constructs and treated for 6 h with either vehicle or 100 μM forskolin, 5 μM butaprost, 100 nM PGE_2, 100 nM PGD_2, or 5 μM BW245C. Cell lysates were assayed for firefly luciferase activity, and values were normalized to Renilla luciferase activity. Values are represented as relative light units and represent means ± SD of triplicate determinations from a representative experiment.

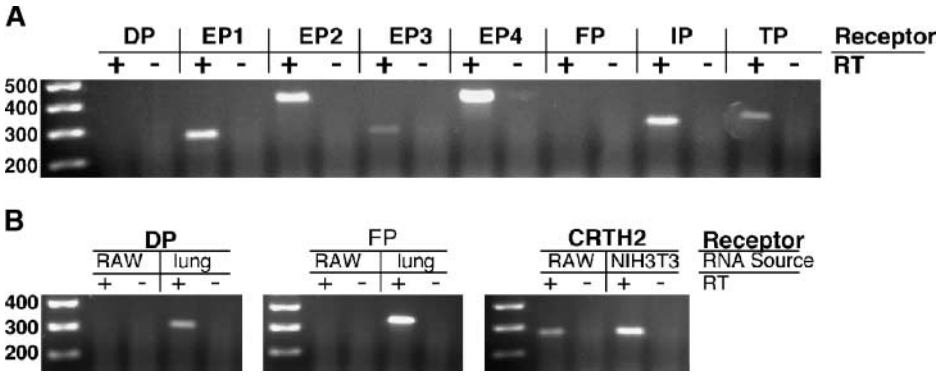

Fig. 6. PG receptor expression in RAW. Total RNA isolated from RAW, NIH 3T3 cells, and mouse lung was subjected to RT-PCR using the gene-specific primers specified in Table 1. Amplification products were resolved by 1.5% agarose gel electrophoresis, and the resulting gels were photographed using the Gel Doc EQ system, as described in Experimental Procedures. Receptor identities are as follows: DP is the prostaglandin D_2 receptor (DPI). EP1-4 are receptors for prostaglandin E_2. FP, IP and TP are the receptors for prostaglandin $F_{2\alpha}$, prostaglandin I_2, and thromboxane A_2, respectively. $CRTH_2$ (chemoattractant receptor homologous molecule on TH2 cells) is the prostaglandin D_2 receptor (DP2).

RAW (PGD_2) is different from that of RPMs (PGI_2), although both macrophage populations produce PGE_2.

The temporal and quantitative differences in PG formation between RPMs and RAW are readily explained by the observed differences in enzyme expression. RAW express much lower constitutive levels of COX-1 than do RPMs, which decreases their ability to respond quickly to LPS and reduces their overall PG synthetic capacity compared with RPMs. This conclusion is supported by our finding that the selective COX-2 inhibitor, SC236, reduced PG synthesis in RPMs by only 33% after 2 h of LPS treatment (24), whereas in RAW, the inhibition was 71% (Fig. 4C). In addition, the induction of COX-2 occurs more slowly in LPS-treated RAW than in RPMs, further contributing to the delay in PG synthesis in RAW. Even though the two cell types reach similar maximal levels of COX-2 expression, the fact that significant increases in RAW COX-2 levels occur much later than the maximum availability of free 20:4 may also reduce the efficiency of PG synthesis in these cells.

It is notable that RAW express much higher levels of $cPLA_2$ than do RPMs; however, this is not reflected in comparably higher levels of free 20:4. In fact, although 20:4 levels in total RAW cultures are somewhat increased compared with those in RPMs, actual cell-associated 20:4 (the pool of 20:4 directly available to COX for PG synthesis) appears to be similar in the two macrophage populations. The failure of RAW to achieve higher levels of free 20:4 commensurate with their higher $cPLA_2$ expression may be attributable to the failure of complete activation of the $cPLA_2$ enzyme, which requires increased intracellular Ca^{2+} and/or phosphorylation (35–44). Alternatively, increased deesterification by $cPLA_2$ may be compensated by a more rapid rate of reesterification of 20:4 into phospholipids.

Compared with RPMs, RAW produce much higher levels of TNF-α and sustain maximal levels for a longer period of time. This difference is, at least in part, attributable to the lack of suppression of TNF-α synthesis by endogenous PGs in RAW. Our results clearly demonstrate decreased TNF-α synthesis by LPS-treated RAW in the presence of exogenous PGs under conditions that mimic the types and concentrations of PGs produced by RPMs. However, the primary PGs produced by RAW differ from those of RPMs by a substitution of PGD_2 for PGI_2. PGD_2, acting through the DP1 receptor, evokes increases in intracellular cAMP, the second messenger believed to be responsible for TNF-α synthesis inhibition. However, our data show that RAW lack the DP1 receptor, which means that they are incapable of responding to their primary endogenous PG with augmented cAMP levels. RAW can respond to their secondary PG, PGE_2, with increases in cAMP through expression of the EP2 and EP4 receptors. However, our prior studies of RPMs indicate that the inhibition of TNF-α secretion depends on the accumulation of adequate PG levels within the first 2 h of LPS treatment. In RAW, PGE_2 synthesis is delayed, so the lowest possible levels expected to be effective (7 nM) are not reached until 4 h of incubation. Thus, the lack of COX-1, and the slow COX-2 induction in response to LPS, contribute to the failure of PG-mediated TNF-α synthesis regulation in these cells.

Our results show that the response of RAW to LPS is similar in many ways to that of RPMs; however, the exclusive use of RAW as a model of macrophage physiology would lead to a failure to observe some key aspects of the LPS response observed in the primary cells studied here. It is important to note, however, that the degree to which RAW serve as an accurate model for primary macrophages will depend on the primary cell population used for comparison. Thus, it is well established that there are significant qualitative and quantitative differences in 20:4 metabolism between primary macrophages from different tissues of origin and between macrophages after exposure to various inflammatory stimuli. For example, in response to a zymosan stimulus, murine RPMs were found to produce larger quantities of PGs than elicited or activated

peritoneal macrophage populations obtained after intraperitoneal injection of thioglycollate broth, heat-killed *Corynebacterium parvum*, or bacillus Calmette-Guerin (45, 46). More recently, Watanabe, Kobayashi, and Okuyama (47) noted that rat thioglycollate-elicited peritoneal macrophages produced lower quantities of PGE_2 than RPMs in response to calcium ionophore, exogenous 20:4, or LPS. They noted that the elicited cells constitutively expressed lower levels of COX-1 than did RPMs and that the induction of COX-2 protein in response to LPS was also lower in elicited macrophages. Because RAW were originally derived from a pristane-elicited cell population, it is to be expected that they will serve as a better model for elicited macrophages than the resident cells used here for comparison (25).

The finding that RAW produce PGD_2 as their primary PG but lack the DP1 receptor distinguishes them physiologically from both resident and elicited primary peritoneal macrophages. Under most circumstances, elicited macrophage populations produce lower quantities of PGE_2 and PGI_2 than RPMs but approximately the same quantities of thromboxane A_2. However, these cells do not produce PGD_2 as their primary product (45, 46). Therefore, the formation of PGD_2 by RAW is not readily explained by their derivation from elicited macrophages. Rather, we suggest that the switch to PGD_2 formation in RAW is a reflection of the transformed phenotype. Our results indicate that the change in the major PG produced in RAW was not accompanied by a complementary change in receptor expression. Consequently, unlike primary peritoneal macrophages, RAW do not respond to their major endogenous PGs with increases in intracellular cAMP. Therefore, they escape an important mechanism for PG-mediated autocrine regulation that is exhibited by primary peritoneal macrophages. Our data demonstrate that this causes alterations in the regulation of cytokine production. Whether it also contributes to the maintenance of the transformed phenotype is an interesting question for further study.

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